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Clin Cancer Res 2003;9:5018-5027.

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Induction of Apoptosis by Apicidin, a Histone Deacetylase Inhibitor, via the Activation of Mitochondria-Dependent Caspase Cascades in Human Bcr-Abl-Positive Leukemia Cells

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ABSTRACT

Purpose: Apicidin, a histone deacetylase inhibitor, is a novel cyclic tetrapeptide that exhibits potent antiproliferative activity against various cancer cell lines. The aim of this study was to examine the potential of apicidin to induce apoptosis in human Bcr-Abl-positive leukemia cells and to assess the mechanism of apicidin-induced apoptosis.

Experimental Design: Cells were exposed to various concentrations of apicidin for 2–72 h, after which the levels of apoptosis, histone acetylation, mitochondrial damage, caspase activation, and Bcr-Abl expression were assessed.

Results: Apicidin induced apoptosis in K562 cells in a concentration- and time-dependent manner. Similarly, apicidin notably induced the apoptosis in the primary leukemic blasts obtained from chronic myelogenous leukemia patients in blast crisis. The acetylated histone H4 levels increased in a concentration-dependent manner in the K562 cells. However, the timing of cell death caused by apicidin did not exactly correlate with the histone deacetylase inhibitory effect. The disruption of the mitochondrial membrane potential, cytochrome *c* release into the cytosol, and the mitochondrial Bax translocation were notably demonstrated after the apicidin treatment. Apicidin induced the proteolytic cleavage of procaspase-9, -3, -8, and poly(ADP-ribose) polymerase. Pretreatment of the K562 cells with the caspase-3

inhibitor, DEVD-CHO, completely inhibited the apicidin-induced apoptosis, suggesting that apicidin-induced apoptosis was caspase-dependent. The Fas/Fas ligand death receptor pathway was not involved in the apicidin-mediated apoptosis in K562 cells. Pretreatment of the cells with the caspase-9 inhibitor LEHD-fmk abrogated the apicidin-induced cleavage of procaspase-3, -8, and poly(ADP-ribose) polymerase. The p210 Bcr-Abl protein levels were notably decreased after the apicidin treatment, with near complete loss after 48 h. Reverse transcription-PCR assay demonstrated that the Bcr-Abl mRNA level was also remarkably decreased in a time-dependent manner.

Conclusions: These results indicate that apicidin effectively induces the apoptosis of Bcr-Abl-positive leukemia cells through the activation of the mitochondrial pathway-dependent caspase cascades. The down-regulation of Bcr-Abl mRNA might also be one of the mechanisms implicated in the apicidin-mediated apoptosis in the K562 cells. This study provides the rationale to additionally investigate apicidin as a potential therapeutic agent for the drug-resistant Bcr-Abl-positive leukemia cells.

INTRODUCTION

The acetylation and deacetylation of the histones of the core proteins of the nucleosomes in chromatin play an important role in regulating gene expression (1). Transcriptionally active genes are associated with the highly acetylated core histones. In contrast, histone deacetylation is associated with gene silencing and transcriptional repression (2, 3). The acetylation status of the histones is controlled by the activities of two enzyme families, histone acetyltransferases and HDAC³ (4, 5). The deregulation of histone acetylase and HDAC has been suggested to play a causative role in the development of cancer by changing the expression pattern of a variety of genes, such as oncogenes and tumor suppressor genes (6, 7). Gene silencing by the HDAC complexes is an important mechanism in the development of some types of leukemia, most notably APL (8). The two fusion proteins in APL, promyelocytic leukemia-retinoic acid receptor α , and promyelocytic leukemia zinc finger-retinoic acid receptor α , recruit the nuclear corepressor-HDAC complexes. This leads to histone deacetylation and transcriptional repression, which

Received 12/26/02; revised 6/11/03; accepted 6/24/03.

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Supported in part by 1998 Research Grant of Institute of Genetic Science provided by Hanmi Pharmaceutical Co. (to Y. H. M.) and 2002 Research Grant of Institute of Genetic Science provided by Yuhan Corporation. (to Y. H. M.).

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³ The abbreviations used are: HDAC, histone deacetylase; APL, acute promyelocytic leukemia; AML, acute myeloid leukemia; CML, chronic myelogenous leukemia; ALL, acute lymphoblastic leukemia; PARP, poly(ADP-ribose) polymerase; MMP, mitochondrial membrane potential, $\Delta\psi_m$; PI, propidium iodide; DiOC₆, 3,3-dihexyloxycarbocyanine iodide; RT-PCR, reverse transcription-PCR; IL, interleukin; DEVD-CHO, N-acetyl-Asp-Glu-Val-Asp-aldehyde; PBS-T, phosphate buffered saline-Tween; TBST, Tris buffered saline Tween.

results in a maturational block in the myeloid cell lineage (8, 9). There is also evidence implicating aberrant HDAC-mediated transcriptional repression in some forms of AML. The AML-ETO fusion protein, often demonstrated in the M2 subtype of AML with t(8;21) translocation, is a potent transcriptional repressor by recruiting HDAC to the transcriptional repressor complex (10, 11).

In recent years, an increasing number of structurally diverse HDAC inhibitors, which accumulate the acetylated histones in the nucleus, have been demonstrated to induce growth arrest, differentiation, and apoptosis of tumor cells both *in vitro* and *in vivo* (11–16). Accumulating evidence suggests that the induction of histone hyperacetylation by the HDAC inhibitors is responsible for the antiproliferative activity along with the reversal of the neoplastic characteristics through the selective transcriptional activation of certain genes, which play important roles in the cell cycle and cell morphology (5–7, 14, 17). The effect of HDAC inhibition and its potential clinical utilization have been reported in AML (18–20). Treatment with an HDAC inhibitor can relieve the ETO-mediated transcriptional repression and induce AML-ETO cell differentiation (11). It was reported that sodium phenylbutyrate, an HDAC inhibitor, induced complete remission in an APL patient with all-*trans* retinoic acid resistance (20). The antileukemia effects of the HDAC inhibitors in APL were caused primarily by the initiation of leukemic cell differentiation, which is similar to the proposed all-*trans* retinoic acid effect (21, 22). However, a recent study demonstrated that the HDAC inhibitors can induce caspase-dependent apoptosis and the down-regulation of *daxx* in APL cells with t(15;17; Ref. 23). Apoptosis was also demonstrated in lymphoblasts and myelomonocytic leukemia cells after treatment with HDAC inhibitors (24, 25). This suggests the potential utility of HDAC inhibitors in treating leukemic disorders.

K562 is a CML cell line expressing a p210 Bcr-Abl fusion protein. Previous studies have shown that this cell line is particularly resistant to cell death via apoptosis, irrespective of the stimuli (26, 27). STI571, a new Abl tyrosine kinase inhibitor, has been shown to inhibit the growth of Bcr-Abl-positive leukemia cells *in vitro* (28) and is highly effective in treating the early stages of CML (29). However, the emergence of STI571 resistance in CML patients, who were initially responsive to this agent (30, 31), has prompted the search for an alternative approach to treating this disease using HDAC inhibitors.

Apicidin is a novel HDAC inhibitor with a potent broad spectrum of antiproliferative activity against various cancer cell lines (14, 24). It was reported that apicidin arrests human cancer cell growth by selectively inducing p21^{WAF/Cip1}. Apicidin has been demonstrated to potentially induce HL-60 leukemia cell apoptosis through the selective induction of the Fas/Fas ligand, resulting in the mitochondrial release of cytochrome *c*, and the subsequent activation of caspase-9 and caspase-3 (32). If the apicidin treatment can effectively induce Bcr-Abl-positive CML cell apoptosis and its apoptosis-inducing mechanisms be understood, the development of novel therapeutic approaches for treating Bcr-Abl-positive leukemia, such as CML and a subset of ALL, is expected. In this report, apicidin is demonstrated to induce the apoptosis of Bcr-Abl-positive leukemia cells through the activation of a mitochondria-dependent caspase cascade. Because apicidin induces the cell death in apoptosis-resistant

Bcr-Abl-positive leukemia cells efficiently, its potential as a therapeutic agent should be additionally validated.

MATERIALS AND METHODS

Cells and Culture Condition. The K562 cell line was obtained from the American Type Culture Collection (Manassas, VA). The cells were grown in RPMI 1640 (Life Technologies, Inc. Grand Island, NY) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, UT), 1% penicillin/streptomycin, and 2 mM L-glutamine (Life Technologies, Inc.). The cultures were maintained in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. In all of the experiments, 2×10^6 cells/ml of the growing cells were placed in each well of a six-well plate containing 3-ml RPMI 1640 plus 10% fetal bovine serum and were exposed to apicidin for the indicated time intervals. Two days before commencing the STI571 (Gleevec; Novartis, Basel, Switzerland), the peripheral blood leukemic blasts were prepared from 4 CML patients in blast crisis, who provided informed consent. The peripheral blood was sedimented on Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) density gradient. After washing the cells collected from the upper interface, T-cell depletion was performed using a high gradient magnetic cell separation system/anti-CD3 monoclonal antibody (Miltenyi Biotech, Auburn, CA) according to the manufacturer's instructions. A morphological evaluation indicated that >95% of the isolated cells were leukemic blasts.

Reagents and Antibodies. Apicidin [cyclo(*N*-O-methyl-L-tryptophanyl-L-isoleucinyl-D-pipecolinyl-L-2-amino-8-oxodecanoyl)] was purchased from Calbiochem (San Diego, CA). A stock solution (1 mM) in sterile DMSO (Sigma-Aldrich, St. Louis, MO) was stored at –80°C for no more than 3 months. The final DMSO concentration in the cultures was kept at 0.1%, which is a concentration that is nontoxic to the cells. A caspase-3 inhibitor, DEVD-CHO (Calbiochem), and a caspase-9 inhibitor, LEHD-fmk (Calbiochem), were dissolved in DMSO, and stored at –70°C until needed. The NOK-1 monoclonal antibody that interferes with the Fas-Fas ligand interaction and inhibits Fas signaling was obtained from PharMingen (San Diego). The antibodies against procaspase-3, procaspase-8, and procaspase-9 were obtained from PharMingen. The antibodies against the cleaved caspase-3, cleaved caspase-8, cleaved caspase-9, cytochrome *c*, Fas ligand, Bax, and PARP were purchased from Cell Signaling Technology (Beverly, MA). The antibodies against Bcl-X_L, Bcl-2, c-Abl, and Fas were obtained from Santa Cruz Biotechnology Inc (Santa Cruz, CA). The antibodies against histone H4, α -tubulin, and acetyl-histone H4 were purchased from Upstate Biotechnology Incorporated (Lake Placid, NY).

Annexin V Staining. The Annexin V assays were performed according to the manufacture's protocol (PharMingen). Briefly, the cultured cells were collected, washed with binding buffer, and incubated in 200 μ l of a binding buffer containing 5 μ l of Annexin-V-FITC. The nuclei were counterstained with PI. The percentage of apoptotic cells was determined using FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Cell Cycle Analysis. The cells were washed twice with PBS and fixed with 70% ethanol/PBS. They were then treated with 0.5 mg/ml RNase (Sigma) in PBS with 0.1% saponin, and incubated at 37°C for 30 min before staining with 20 µg/ml PI for 30 min at 4°C. The cells (1×10^6) were then analyzed for their DNA content using a FACSCalibur flow cytometer equipped with CellQuest software (Becton Dickinson Immunocytometry Systems).

DNA Fragmentation Assay. The cell pellets (1×10^6 cells) were resuspended in 500 µl of a lysis buffer [0.5% Triton X-100, 10 mM EDTA, and 10 mM Tris-HCl (pH 8.0)] at room temperature for 15 min and centrifuged at $16,000 \times g$ for 10 min. The DNA was then extracted twice with phenol:chloroform (1:1), precipitated with ethanol, and resuspended in a Tris/EDTA buffer [10 mM Tris-HCl (pH 8.0) and 1 mM EDTA]. The DNA was analyzed after being separated by 1.5% agarose gel electrophoresis.

Assessment of Loss of MMP. The MMP was monitored using DiOC₆ as described previously (33). For each condition, 4×10^5 cells were incubated for 15 min at 37°C in 1 ml of 40 nM DiOC₆ (Calbiochem) and subsequently analyzed using a FACSCalibur flow cytometer and CellQuest software. The control experiments documenting the loss of MMP were performed by exposing the cells to 5 µM of carbamoyl cyanide m-chlorophenylhydrazone (Sigma), which is an uncoupling agent that abolishes the MMP.

Histone Isolation and Immunodetection of Acetylated Histone H4. The histones were isolated from the K562 cells after treating them with apicidin for the indicated times using the established techniques (14). The samples were then resuspended in a Laemmli sample buffer for 3 min and subjected to 15% SDS-PAGE to determine the acetylated histone H4 levels.

Preparation of Mitochondria and Cytosolic Fractions. The K562 cells were harvested and rinsed with ice-cold PBS, and the cell pellets were resuspended in 300 µl of buffer A [20 mM HEPES-KOH (pH 7.5), 10 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, and 1 mM DTT] containing 250 mM sucrose and the proteinase inhibitors. After homogenization, the unbroken cells, a sample of the large plasma membrane, and the nuclei were removed by centrifugation at $1,000 \times g$ for 1 min at 4°C. The supernatant was then centrifuged at $10,000 \times g$ for 20 min at 4°C. The pellet fraction containing the mitochondria was dissolved in 50 µl of the TNC buffer [10 mM Tris acetate (pH 8.0), 0.5% NP40, and 5 mM CaCl₂]. The supernatant was additionally centrifuged at $50,000 \times g$ for 2 h to obtain the cytosol, which was immediately subjected to Western blot analysis.

Western Blot Analysis. After the treatment, whole cell pellets (1×10^7 cells/condition) were washed twice in PBS, resuspended in 50 µl of PBS, lysed by adding 50 µl of a 2 × Laemmli buffer [$1 \times = 30$ mM Tris-base (pH 6.8), 2% SDS, 2.88 mM β-mercaptoethanol, and 10% glycerol], and briefly sonicated. The homogenates were quantified using a Coomassie protein assay reagent (Pierce, Rockford, IL). Equal amounts of protein (20 µg) were separated by SDS-PAGE and electroblotted to nitrocellulose. The blots were stained in 0.1% amido black and destained in 5% acetic acid to ensure the transfer and equal loading. After blocking in PBS-T (0.05%) and 5% milk for 1 h at 22°C, the blots were incubated in a fresh blocking solution with an appropriate dilution of the primary antibodies

for 4 h at 22°C. The blots were washed 3×5 min in PBS-T and then incubated with a 1:2000 dilution of horseradish peroxidase-conjugated secondary antibody (Bio-Rad Laboratories, Hercules, CA) for 1 h at 22°C. After washing four times in TBST, the reactive proteins were visualized using an enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ).

RT-PCR Analysis. The total cellular RNA was isolated from the apicidin-treated cells using an RNeasy mini kit (Quiagen, Hilden, Germany). Using equal amounts of the RNA from each time point, the cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (Perkin-Elmer, NJ). A two-step nested PCR procedure was performed using three sets of primers. The primer sets used for the first RT-PCR were: B1 5'-GAAGTGTTCAGAAGCTTCTCC-3'; B3 5'-ACCATCGTGGGCGTCCGCAAGA-3'; and A3 5'-TGATTATAGCCTAAGACCCGGA-3'. The primer sets used for the nested PCR were: A4 5'-ATCTCCACTGGCCACAAAATCAT. ACA-3'; B2 5'-TGGAGCTGCAGATGCTGACCAACTCG-3'; and B4 5'-AG-ATCTGGCCCA ACGATGGCGAGGGC-3'. Gel electrophoresis and ethidium bromide staining was used to visualize the PCR products

RESULTS

Induction of Apoptosis in Bcr-Abl-Positive Leukemia Cells by Apicidin.

This study demonstrated that apicidin induced apoptosis in the K562 cells in a concentration- and time-dependent manner. As shown in Fig. 1A, treating the K562 cells with 0.1 µM apicidin for 72 h caused only a slight increase in the proportion of apoptotic cells. However, increasing the apicidin concentration (1.0–20 µM) for 48–72 h resulted in a sharp increase in the proportion of apoptotic cells, suggesting that cell death occurred in a dose-dependent manner. When the K562 cells were exposed to 2.5 µM apicidin, the proportion of apoptotic cells increased in a time-dependent manner. Almost 90% of the cell population underwent apoptosis after 72 h, compared with ≤10% of the control (0.1% DMSO-treated) cells (Fig. 1A). The cells treated with apicidin showed the typical apoptotic features such as cell shrinkage, chromatin condensation, and nuclear fragmentation (data not shown). An analysis of the DNA fragmentation showed an increase in cellular DNA degradation in a time-dependent manner (Fig. 1B), which is consistent with the increased proportion of apoptotic cells. Similar results were obtained when the apoptosis was monitored by cell cycle analysis. Exposure to 2.5 µM apicidin for 8 h had no remarkable effect on the cell cycle distribution of the K562 cells. However, the apicidin treatment for 24–72 h resulted in a progressive increase in the sub-G₁ cell fraction (Fig. 1C). Similarly, the apicidin treatment remarkably increased the levels of apoptosis in the primary leukemic blasts obtained from the 4 patients with CML in blast crisis in a time-dependent manner (Fig. 1D).

Effects of Apicidin on Histone Acetylation. The effects of apicidin on the intracellular level of histone H4 was analyzed to additionally examine the relationship between cell death and histone acetylation in the K562 cells. The cells were treated with various apicidin concentrations for the indicated times, and the histones extracted from the nuclei

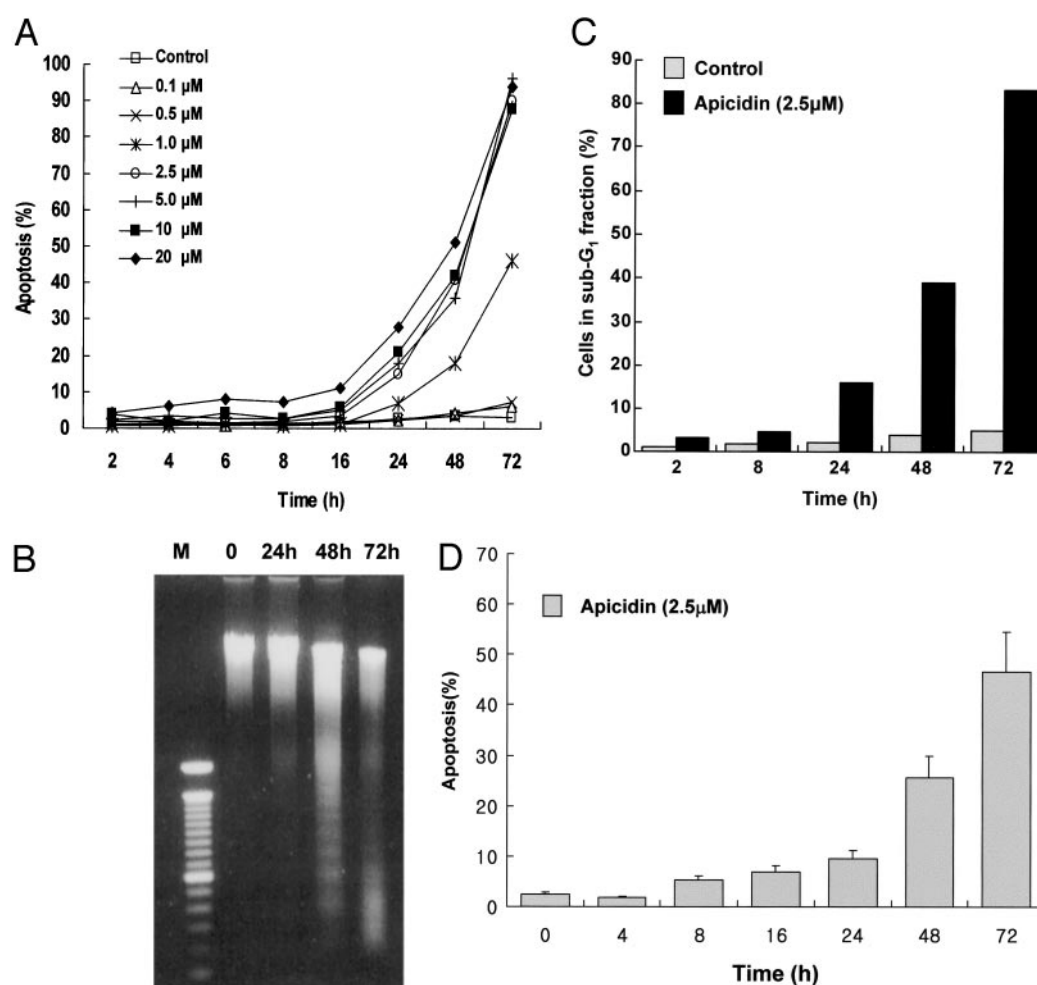


Fig. 1 Apicidin-induced apoptosis in Bcr-Abl-positive leukemia cells. **A**, proliferating K562 cells ($2 \times 10^6/\text{ml}$) were exposed to the designated concentrations of apicidin for the indicated times, after which the percentage of apoptotic cells was determined by flow cytometric analysis, as described in "Materials and Methods." The data are expressed as a mean value of the percentage of apoptotic cells from three independent experiments performed in duplicate. **B**, the K562 cells were exposed to 2.5 μM apicidin up to 72 h, after which the total genomic DNA was extracted and resolved on 1.5% agarose gel. The apoptotic DNA fragmentation was visualized by ethidium bromide staining and photographed under UV illumination. Lane M, DNA marker. **C**, at the indicated time points after treatment with 2.5 μM apicidin, the cells were harvested and fixed in 70% ethanol. After staining with PI, the apoptotic DNA content was analyzed by flow cytometry. The number of apoptotic cells in the sub-G₁ fraction is expressed as a percentage of the total number of cells. **D**, primary leukemic blasts ($2 \times 10^6/\text{well}$) obtained from 4 patients with CML in blast crisis were exposed to 2.5 μM of apicidin for the indicated times, after which the percentage of apoptotic cells was determined by flow cytometric analysis. The data represents the means of the four separate experiments performed in duplicate; bars, \pm SD.

were subjected to Western blot analysis. As shown in Fig. 2A, the amount of histone H4 acetylation increased in a concentration-dependent manner, reaching a maximum level at 2.5 μM apicidin, which remained at this level at higher concentrations. Apicidin did not cause any change in the amount of histone H4 (Fig. 2A). The amount of histone H4 acetylation notably increased as early as 2 h after the treatment with 2.5 μM of apicidin, with a remarkable increase in the acetylated histone H4 levels after 24 h (Fig. 2B).

Disruption of MMP by Apicidin. The amount of MMP disruption was assessed after the apicidin treatment by measuring the mitochondrial uptake of a membrane potential-sensitive dye, DiOC₆, in the K562 cells. When the cells were treated with 2.5 μM apicidin, the population that lost MMP was 32.8%

at 16 h and 51.7% at 24 h (Fig. 3A). This suggests that an apoptosis-inducing mechanism triggered by apicidin operates via the mitochondria in the K562 cells.

Effect of Apicidin on Cytochrome *c* Release and Bax Translocation. Fig. 3B shows the time-dependent release of cytochrome *c* from the mitochondria into the cytosol after being exposed to 2.5 μM apicidin. Increased levels of cytochrome *c* were notably detected in the cytosol after 16 h, reaching the maximum levels after 24 h of treatment. Cytochrome *c* in the mitochondrial fraction decreased in parallel after 16 h of apicidin treatment. No significant changes in the Bcl-2 or Bcl-X_L protein levels were found in the K562 cells after the apicidin treatment (data not shown). Increasing amounts of the Bax protein notably detected in the mitochondrial fraction early after

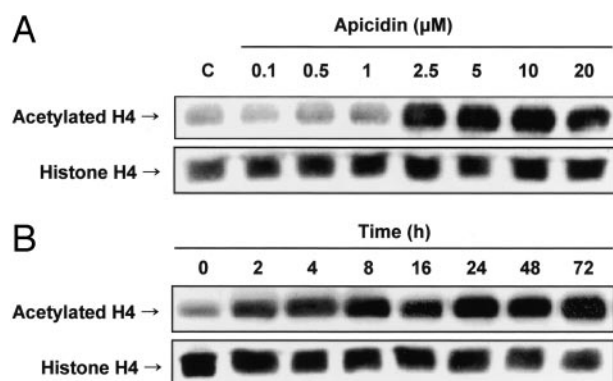


Fig. 2 Effect of apicidin on the acetylation of histone H4 in K562 cells. The K562 cells were either exposed to various apicidin concentrations for 24 h (A) or treated with 2.5 μ M apicidin for the indicated times (B). The histone fractions were isolated, and 10 μ g of the protein were run on 15% SDS-PAGE gel, blotted, and probed with antibodies against acetylated histone H4 and α -tubulin, which served as an internal control. In addition, the histone H4 expression level in the cell lysate was examined by Western blot analysis using anti-histone H4 antibody. c, control (0.1% DMSO-treated) cells.

8 h of apicidin treatment (Fig. 3C). The cytosolic Bax protein level decreased after 16 h of apicidin treatment.

Processing of Caspase-3, -8, and -9 by Apicidin. The processing of the caspase cascades was investigated. As shown in Fig. 4A, incubation of the K562 cells (2–72 h) with 2.5 μ M of apicidin resulted in the proteolytic cleavage of procaspase-3 to an active M_r 19,000 cleaved product. This indicated that the effector caspase-3 was activated during apicidin-induced apoptosis. The procaspase-3 cleavage was demonstrated after 24 h of treatment. The increased levels of cleaved PARP (M_r 89,000 and M_r 24,000) were observed in proportion to the processing of caspase-3 (Fig. 4A). To confirm the involvement of caspase-3 in apicidin-induced apoptosis, the effect of DEVD-CHO, a caspase-3 inhibitor, on the apicidin-induced apoptosis, and the cleavage of PARP and procaspase-3 was examined. As shown in Fig. 4B, pretreatment the K562 cells with 50 μ M of DEVD-CHO for 2 h completely inhibited the apicidin-induced apoptosis as well as the cleavage of procaspase-3 and PARP. This indicates that apicidin induces the apoptosis of K562 cells in a caspase-dependent manner. As shown in Fig. 4A, the cleavage of procaspase-9 into the M_r 35,000 product was induced by apicidin concomitantly with that of procaspase-8 and -3. Pretreatment of the K562 cells with 100 μ M of the caspase-9 inhibitor LEHD-fmk abrogated the apicidin-induced processing of caspase-9, -3, -8, and PARP (Fig. 5).

Apicidin-Induced Apoptosis Occurs Independently of Fas Receptor System. As shown in Fig. 6A, both the Fas receptor and the Fas ligand expression levels were unchanged in the K562 cells after being treated with 2.5 μ M apicidin. In addition, it was found that preincubation with 0.6 μ g/ml of the Fas-blocking NOK-1 monoclonal antibody for 2 h did not block the apicidin-induced apoptosis and caspase-3 activation in the K562 cells (Fig. 6B).

Down-Regulation of p210 Bcr-Abl Protein during Apicidin-Induced Apoptosis. The changes in the level of the Bcr-Abl molecule after treatment with 2.5 μ M apicidin treatment

were evaluated because apicidin induced apoptosis in the Bcr-Abl-positive leukemia cells. As shown in Fig. 7A, the p210 Bcr-Abl protein levels were reduced after 16 h of apicidin treatment, with a near complete loss of the Bcr-Abl protein after 48 h of apicidin treatment. Bcr-Abl mRNA expression was also examined in the treated cells. The RT-PCR assay demonstrated that the Bcr-Abl mRNA (305 bp) expression level was also reduced after the apicidin treatment in a time-dependent manner (Fig. 7B).

DISCUSSION

This study demonstrated that apicidin induces apoptosis in human Bcr-Abl-expressing leukemia cells, including the primary leukemic blasts obtained from CML patients in blast crisis, in a time- and concentration-dependent manner. The K562 cells exposed to apicidin demonstrated a dramatic increase in the mitochondrial dysfunction (e.g., loss of MMP and cytosolic cytochrome *c* release) associated with the activation of the caspase cascades, PARP cleavage, and the appearance of the characteristic morphological features of apoptosis.

Accumulating evidence suggests that the inhibition of histone deacetylation is closely related to the induction of apoptosis in various cancer cells, including APL (23) and myelomonocytic leukemia (24, 25). We found that, using acetylated histone type-specific antibody, acetylated histone H4 accumulated in the K562 cells shortly after the apicidin treatment. However, the timing of cell death caused by the apicidin did not exactly correlate with the HDAC inhibitory effects. There was a notable time lag between the initiation of the increase in the acetylated histone H4 levels and the induction of apoptosis in the K562 cells. In addition, the extent of HDAC inhibition and apoptosis were not closely correlated. The phenotype induced by the HDAC inhibitors, e.g., apoptosis, appears to be related to the intrinsic characteristics of the particular transformed cell line rather than to the extent of HDAC inhibition itself (17). It was reported that sodium butyrate and trichostatin readily induced apoptosis in the human IL-2-dependent ILT-Mat leukemia cells, whereas they induced far less apoptosis in the K562 cells (34). However, these inhibitors similarly increased the acetylation levels of histone in both cell types (34). The present data suggest that histone hyperacetylation alone does not account for the apoptotic effect, and different mechanisms other than histone acetylation could be involved in the HDAC inhibitor-mediated apoptosis in Bcr-Abl-positive leukemia cells.

Two pathways of caspase activation for the induction of apoptosis were identified. The first begins at the death receptors such as Fas (35). When a Fas ligand binds to the Fas death receptor, the adaptor molecule, FADD, is recruited to the receptor. This allows the binding and proteolysis of procaspase-8, which generates activated caspase-8 (36–38). The activated caspase-8 then processes the effector caspases (caspase-3, -6, and -7) for activation (35, 39, 40). In the second pathway, diverse proapoptotic signals converge at the mitochondrial level, inducing the translocation of cytochrome *c* into the cytosol. Cytochrome *c* triggers caspase-9 activation initiating a downstream caspase cascade through the complex formation with Apaf-1, dATP, and pro-caspase-9 in the cytosol, which ultimately leading to the activation of the executioner caspase-3 and

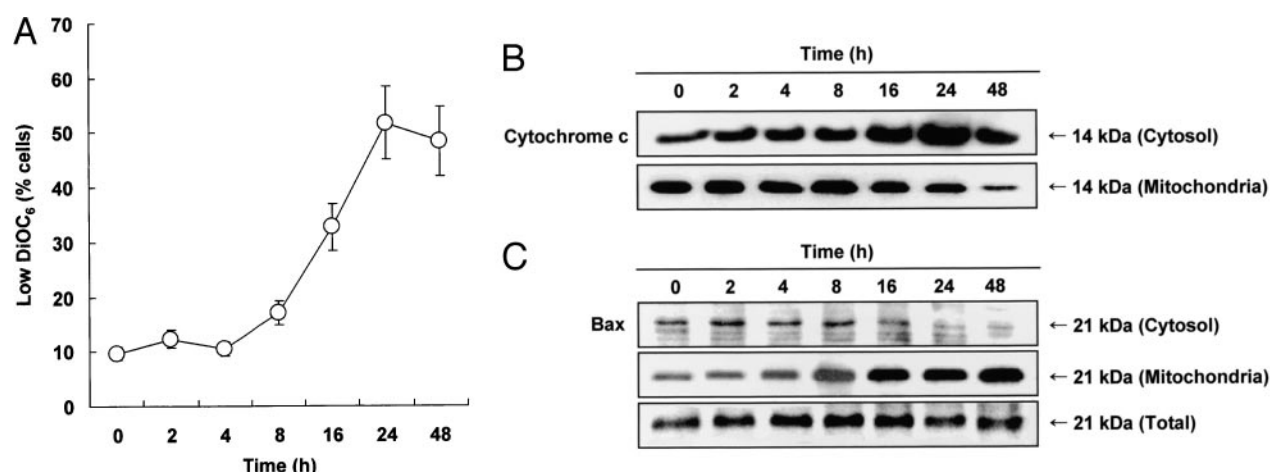


Fig. 3 Apicidin induces the mitochondrial membrane damage. **A**, K562 cells were exposed to 2.5 μ M of apicidin for the indicated times, after which the loss of the mitochondrial membrane potential ($\Delta\psi_m$) was assessed in the DiOC₆-treated specimens by flow cytometry, as described in "Materials and Methods." The values are expressed as the mean percentage of cells exhibiting a low MMP, as reflected by reduced levels of DiOC₆ uptake; bars, \pm SD. Effect of apicidin on the cytochrome *c* release (**B**) and Bax translocation (**C**). The K562 cells were treated with 2.5 μ M of apicidin for the indicated times, after which the cytosolic and mitochondrial fractions were prepared as described in "Materials and Methods" and separated by SDS-PAGE. Equal amounts of the protein (20 μ g) from the cytosolic and mitochondrial fractions were analyzed by Western blotting using antibodies against cytochrome *c* and Bax, respectively. The results of a representative study are shown; two additional experiments produced equivalent results.

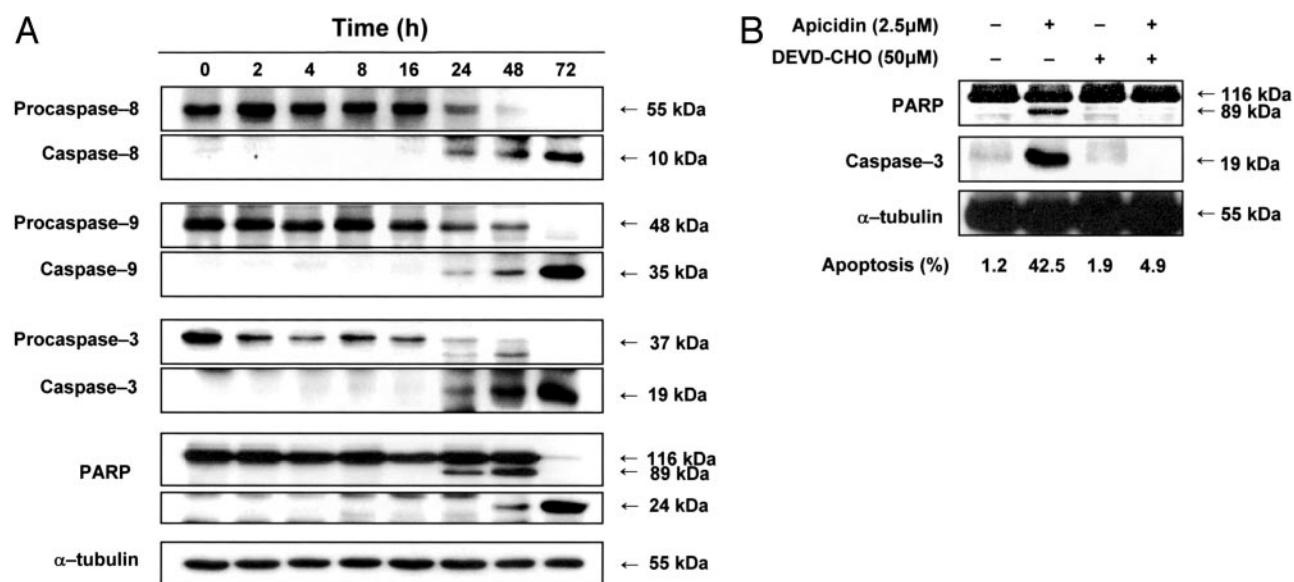


Fig. 4 Apicidin-induced processing of caspase-8, -9, -3, and PARP. **A**, the K562 cells were exposed to 2.5 μ M of apicidin for the indicated times, after which the protein fractions were subjected to Western blot analysis. After separation on SDS-PAGE, the blots were probed with antibodies against procaspase-8, cleaved caspase-8, procaspase-9, cleaved caspase-9, procaspase-3, cleaved caspase-3, and PARP. Each lane was loaded with 20 μ g of protein, and after analysis, the blots were stripped and reprobed for α -tubulin to ensure an equal loading and protein transfer. The results of a representative study are shown; two additional experiments produced equivalent results. **B**, effect of caspase-3 inhibitor on the apicidin-induced apoptosis and processing of caspases. The K562 cells were pretreated with 50 μ M of the caspase-3 inhibitor DEVD-CHO for 2 h and then treated with 2.5 μ M apicidin for 48 h. The processing of procaspase-3 or PARP was examined by Western blot analysis. The mean value of the percentage of apoptotic cells determined by flow cytometry is shown in the bottom line.

finally cell death (39–44). In this study, the release of cytochrome *c* into the cytosol was demonstrated shortly after being exposed to apicidin, with maximum levels being observed after 16 h of treatment. The degree of MMP disruption correlated with the release of cytochrome *c*. The release of cytochrome *c* was followed by the processing of caspase-3, -8, and -9, indi-

cating that the cytochrome *c* release is a preceding event for the activation of the caspase cascades. Pretreating the K562 cells with a caspase-3 inhibitor completely inhibited the apicidin-induced apoptosis, indicating that the apoptosis process was caspase-dependent.

The processing of caspase-8, -9, and -3, and PARP cleav-

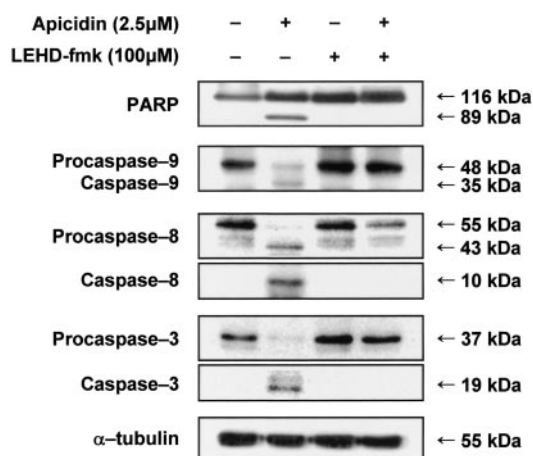


Fig. 5 Effect of caspase-9 inhibitor on the apicidin-induced processing of caspases. The K562 cells were pretreated with 100 μM of caspase-9 inhibitor LEHD-fmk for 2 h, followed by 2.5 μM apicidin treatment for an additional 48 h. The cleavage of procaspase-8, -3, -9, and PARP was examined by Western blot analysis using the corresponding antibodies. The results of a representative study are shown; two additional experiments yielded similar results.

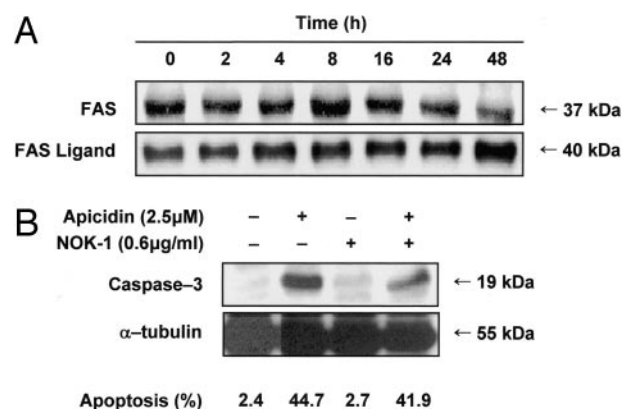


Fig. 6 Apicidin-induced apoptosis in K562 cells is independent of the Fas-Fas ligand pathway. **A**, after treating the K562 cells with 2.5 μM of apicidin for the indicated times, the expression of Fas and the Fas ligand were examined by Western blot analysis using the appropriate antibodies. **B**, the cells were pretreated with 0.6 μg/ml anti-Fas blocking NOK-1 antibodies for 2 h, which was followed by a treatment with 2.5 μM apicidin for an additional 48 h. The cleavage of procaspase-3 was examined by Western blot analysis. The mean value of the percentage of apoptotic cells determined by flow cytometry is shown in the bottom line.

age was detectable after 24 h of apicidin treatment, when the apoptotic cell population and DNA laddering were obvious, albeit only modest, and the maximum levels of H4 acetylation was observed. We observed that the expression levels of both the Fas receptor and the Fas ligand remained unchanged in the K562 cells after the apicidin treatment. It was also found that pretreating the K562 cells with NOK-1 anti-Fas blocking antibody did not block either the apicidin-induced apoptosis or procaspase-3 cleavage. However, in the HL-60 cells, the Fas signaling pathway was directly involved in apicidin-mediated

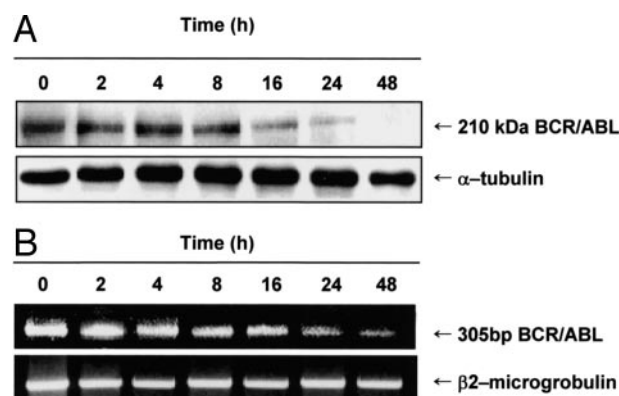


Fig. 7 Effect of apicidin on the Bcr-Abl protein and mRNA expression. **A**, after treating the K562 cells with 2.5 μM apicidin for the indicated times, the p210 Bcr-Abl protein expression was examined by Western blot analysis using c-Abl antibody. Each lane was loaded with 20 μg of protein; the blots were subsequently stripped and reprobed for the expression of α-tubulin to ensure the equivalent loading and transfer. **B**, the Bcr-Abl mRNA expression level was simultaneously determined by RT-PCR, as described in "Materials and Methods." The RT-PCR assay demonstrated that the Bcr-Abl mRNA (305 bp) expression level decreased after the apicidin treatment in a time-dependent manner. β2-Microglobulin was used as the loading control.

apoptosis (32). The effect and mechanism of the HDAC inhibitors on the induction of apoptosis can differ according to the tumor cells and types of HDAC inhibitors. Considering these results, processing of caspase-8 by apicidin was not directly associated with the activation of the Fas/FasL death receptor signaling pathway in Bcr-Abl-positive K562 cells. This study found that pretreating the K562 cells with a specific caspase-9 LEHD-fmk inhibited the apicidin-induced activation of caspase-3, -8, and the induction of apoptosis. These findings favor a model where both caspase-3 and caspase-8 are activated by a common preceding event, *i.e.* mitochondria-dependent procaspase-9 activation in the apicidin-treated K562 cells.

Bax translocation from the cytosol into the mitochondria after apoptotic stimulation is believed to be a crucial step for triggering the release of cytochrome *c* release from the mitochondria and disrupting MMP (45, 46). In the present study, Bax translocation into the mitochondria preceded the maximum degree of cytochrome *c* release into the cytosol and remained elevated thereafter. The increase in the mitochondrial Bax levels was succeeded by a decrease in the cytosolic Bax protein levels. It was reported recently that the sphingolipid metabolite phyto-sphingosine induced the mitochondrial translocation of Bax without changing the Bax protein levels in the cancer cell lines (47). This suggests that an intracellular process involved in the transmembrane localization of the Bax protein can differ according to the cell types as well as the apoptosis-inducing agents. Because there was no p53 protein expression in the K562 cells in this study (data not shown), the apicidin-induced Bax translocation can be considered to be p53-independent. Because the high Bcr-Abl expression level prevents the translocation of cytosolic Bax and the release of cytochrome *c* into the cytosol (48), the prominent increase in the amounts of Bax translocation and cytochrome *c* released into the cytosol might

be closely associated with the down-regulation of the Bcr-Abl protein.

The K562 cell line displays a relatively high level of resistance to most cytotoxic drugs, which is probably because of a combination of Bcr-Abl oncogene expression (26, 49–51). The resistance to chemotherapeutic agent-induced apoptosis in the K562 cells is known to be associated with the inhibition of MMP collapse and the release of cytochrome *c*, which prevents the activation of the downstream caspases and apoptosis (50, 52, 53). In addition to the upstream block, it was also found that the K562 cells were resistant to the cytochrome *c*-induced activation of caspase-9 and -3 (54, 55). Because apicidin effectively induced apoptosis in the K562 cells, it is possible that the down-regulation of the p210 Bcr-Abl protein might have taken place during the process of apoptosis. In this study, the Bcr-Abl protein levels reduced considerably after 16 h of apicidin treatment, with a near complete loss after 48 h. The down-regulation of Bcr-Abl correlated with the increase in the cytosolic cytochrome *c* levels, the extent of Bax translocation, the extent of MMP disruption, the activation of the caspase cascade, and the amount of apoptosis in the K562 cells.

RT-PCR analysis demonstrated that the Bcr-Abl mRNA expression levels also decreased significantly after the apicidin treatment. This suggests that apicidin down-regulates the Bcr-Abl molecule at the transcriptional level, although another mechanism, *e.g.*, proteasomal- or caspase-dependent protein degradation, might also be involved in this process. Usually, the HDAC inhibitors can induce transcriptional activation of a number of genes (6, 7, 14, 24, 32). However, the HDAC inhibitors can also down-regulate the transcription of certain genes. Sodium butyrate and trichostatin abrogated the IL-2-mediated gene expression in the IL-2-dependent leukemia cell line (34). FK228 inhibits the induction and activity of hypoxia-inducible factor-1 and the vascular endothelial growth factor in response to hypoxia (56). FR901228 inhibits catalytic subunit of telomerase mRNA expression in the small-cell lung cancer cell lines (57). It was reported recently that suberoylanilide hydroxamic acid, a HDAC inhibitor, also reduce the level of Bcr-Abl mRNA (58). This suggests that the HDAC inhibitors can down-regulate the transcription of specific genes in various cancer cells. However, the mechanisms responsible for the transcriptional down-regulation of these genes have not been defined. Although the precise mechanism by which apicidin down-regulates the Bcr-Abl transcript remains to be clarified, this study suggests that the Bcr-Abl mRNA down-regulation contributes, at least in a part, to the apicidin-induced apoptosis in K562 cells.

These results suggest that the HDAC inhibitor apicidin effectively induces the apoptosis of human Bcr-Abl-positive leukemia cells via the activation of the mitochondrial pathway-dependent caspase cascades. Although the amount of histone H4 acetylation was increased significantly as a result of the apicidin treatment, its direct contribution to apoptosis in the K562 cells was not plausible. The down-regulation of Bcr-Abl mRNA may be one of the important mechanisms involved in the apicidin-mediated apoptosis in the K562 cells. This experimental study provides the rationale to ad-

ditionally investigate apicidin as a potential therapeutic agent for Bcr-Abl-positive leukemia cells.

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